Expression and localization of transcription factor Ets-1 in the rat ovary during the estrous cycle and pregnancy

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Objective: To examine the expression and localization of Ets-1 in the rat ovary during the estrous cycle and pregnancy, and to investigate its effects on ovarian function.
Design: Prospective, randomized study.
Setting: Department of Physiology at Harbin Medical University.
Animal(s): Pubertal female Wistar rats.
Intervention(s): Vaginal smears were taken daily from female rats to determine the stage of the estrous cycle. Pregnancies were achieved by caging female and male rats together overnight. Ovaries were collected from both cycling and pregnant rats for tissue sectioning and RNA and protein extractions.
Main Outcome Measure(s): Real-time quantitative polymerase chain reaction, Western blot, in situ hybridization, and immunohistochemistry were performed to investigate the expression and localization of Ets-1 messenger RNA (mRNA) and protein in the rat ovary during the estrous cycle and pregnancy.
Result(s): During the estrous cycle, the levels of Ets-1 mRNA and protein expression increased during the follicular phase, achieving their highest measurements at proestrus and lowest at metestrus. The expression of Ets-1 mRNA and protein fluctuated during pregnancy, increasing during early pregnancy, then decreasing during mid-pregnancy, and again increasing until parturition. Ets-1 mRNA and protein were present throughout the estrous cycle and pregnancy, principally localized in follicles of various sizes and in the corpus luteum.
Conclusion(s): Ets-1 may participate and play an important role in the regulation of follicular development, corpus luteum formation, maintenance, and regression. (Fertil Steril® 2009;91:1998–2005. ©2009 by American Society for Reproductive Medicine.)

Key Words: Ets-1, follicle, corpus luteum, estrous cycle, pregnancy, rat

The Ets genes are a family of genes comprising more than 30 members, all functioning as transcription factors (1). All Ets factors share an evolutionally conserved DNA-binding domain; the Ets domain, comprising a sequence of 85 amino acids, binds to a consensus DNA sequence centered on the core GGAA/T motif, aptly named the Ets-binding site or PEA3. Ets-1, the first member of the Ets transcription factor family, was first identified as the cellular progenitor of the viral oncogene v-ets in the genome of the avian leukemia retrovirus E26 (2). Originally detected in lymphoid cells of adult tissues (3), the expression of Ets-1 is also observed in a variety of cells, including endothelial cells, vascular smooth muscle cells, lymphocytes, and epithelial cancer cells (4, 5). A large number of studies reported that Ets-1 played a pivotal role in diverse physiologic and pathologic processes, such as cell growth, migration, differentiation, apoptosis, hematogenesis, angiogenesis, osteogenesis, embryogenesis, and tumor invasion (6–10). Another group of closely related studies found that Ets-1 interacted with the promoter regions of these genes, such as urokinase-type plasminogen activator, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase-1, and vascular endothelial growth factor (VEGF) and its receptors, suggesting that it may play a fundamental role in the regulation of the activities of these genes (11–17).

During the estrous cycle and pregnancy, a series of morphologic and functional changes occurs in the rat ovary, coupled with the fluctuation of hormone levels, such as gonadotropin-releasing hormone (GnRH), gonadotrophins, and sex steroids. Numerous cytokines and growth factors, regulated by GnRH and sex steroids, contribute to cell proliferation, apoptosis, angiogenesis, and extracellular matrix degradation. It has been proposed that these factors may be involved in ovarian oocyte maturation, follicular development, ovulation, and corpus luteum (CL) formation and

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regression, such as VEGF, transforming growth factor (TGF)-β, MMPs, and plasminogen activator system (18–22).

Recent data have suggested that Ets-1 is critically involved in female reproduction. Ets-1 was expressed in theca and interstitial cells in adult mouse ovary, in addition to the mammalian and human glandular tubular epithelial cells of the endometrium, during the estrous or menstrual cycle (23). Ets-1 expression was also detected in reproductive system tumors, such as ovarian carcinoma and endometrial cancer (24). Despite numerous studies having suggested the relationship between Ets-1 and female reproduction, there is no clear evidence to suggest the effects of Ets-1 on ovarian function during the estrous cycle and pregnancy. In the present study, we have determined the expression and localization of Ets-1 messenger RNA (mRNA) and protein in rat ovary at various stages during the estrous cycle and pregnancy, so as to investigate the biological function of Ets-1 involved in follicular development, ovulation, corpus luteum formation, maintenance, and regression.

MATERIALS AND METHODS

Animals and Tissue Collection

Pubertal female Wistar rats (aged 2 to 3 months), weighing approximately 200 g, were randomly bred at room temperature (approximately 25°C) in a constant photoperiod (light/dark cycle, 12 hours/12 hours) with free access to water and food. These rats were allowed 7 days to acclimatize to the new surroundings. All animals were handled in accordance with the guidelines for care and use of experimental animals for scientific purposes, and this research was approved by the Harbin Medical University institutional review board.

To determine the stage of the estrous cycle, vaginal smears were taken daily from these animals for at least two consecutive 4-day cycles. Pregnancies were achieved naturally by caging female and male rats together, overnight. The morning of the day on which sperm in female vaginal smears were detected was designated as day 1 of pregnancy, and the first day after parturition was designated P1.

Cycling and pregnant rats were killed at proestrus, estrus, metestrus, and diestrus, on days 1, 3, 5, 7, 9, 18, and 22 of pregnancy, and on P1 (n = 6 per stage). Ovaries were collected and divided into two parts. One part was snap-frozen in liquid nitrogen to be stored at −80°C for RNA and protein extraction and frozen tissue sections. The other parts were immediately fixed and dehydrated for paraffin-embedded sections.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, Ca), in accordance with the manufacturer's instructions, and demi-quantified with an ultraviolet spectrophotometer (Ultrospec 1100 Pro; Amersham, Buckinghamshire, United Kingdom) at an absorbance of 260 nm.

Single-stranded complementary DNA (cDNA) was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa, Otsu, Japan). A reverse transcription reaction system (with a final volume of 20 μL) containing 5 U AMV reverse transcriptase, 4 μL 5× AMV buffer, 2 μL 10 mmol/L deoxyribonucleoside triphosphate mixture, 50 pmol oligo $(dT)_{18}$ primer, 20 U ribonuclease inhibitor, and 1 μg RNA was maintained for 10 minutes at room temperature, followed by incubation at 42°C for 60 minutes and then 95°C for 5 minutes. The cDNA was stored immediately at −20°C until the polymerase chain reaction (PCR).

Real-time quantitative PCR was performed in a 20-μL final reaction volume using an SYBR Premix Ex Taq kit (TaKaRa) according to the manufacturer's protocol. The sequence-specific primers for rat Ets-1 were 5′-CTACCCTTCTGTCATTCTCC-3′ (forward) and 5′-GAGGCGGTC ACAACTATC-3′ (reverse), and the size of the product was 181 base pairs (bp). Rat β-actin was used as internal control (primers were 5′-GTTTGAGACCTT CAACACCCC-3′ and 5′-GTGGCCATCTCTCTTGCTCGA AGTC-3′) with the expected size of 320 bp. The amplification reaction consisted of 10 μmol/L of each primer, 10 μL of 2× SYBR Premix Ex Taq, and 2 μL cDNA template. Real-time PCR was carried out in duplicate for each sample in the LightCycler real-time PCR thermal cycler (Roche, Mannheim, Germany) with capillary system (TaKaRa) using the following parameters: 1 cycle at 95°C for 10 seconds, followed by 40 cycles at 95°C for 5 seconds (denaturation), 60°C for 15 seconds (annealing), and 72°C for 10 seconds (extension). A PCR system devoid of template cDNA was included as negative control. Fluorescence values in each tube were measured at the end of each cycle using the single acquisition mode. Melting curve analysis was performed after the end of the last cycle. The standard curves were generated using average fluorescence values of duplicate standards. Average fluorescence values of samples were then used to calculate the concentration of Ets-1 mRNA in each sample, with LightCycler Data Analysis software. All data were normalized by dividing the amount of Ets-1 by the amount of β-actin used as the control.

In Situ Hybridization

Digoxigenin (DIG)-labeled RNA probes for in situ hybridization analysis were prepared using T7 and SP6 polymerases according to the manufacturer's instructions supplied with a kit (DIG RNA Labeling Kit; Roche). Both sense and antisense probes were routinely used.

The localization of Ets-1 mRNA in frozen tissue sections was detected by in situ hybridization. The process of in situ hybridization was performed as described previously (25). Sections of rat ovaries (5 μm) were hybridized with DIG-labeled antisense or sense rat Ets-1 complementary RNA (cRNA) probes. The frozen sections on poly-L-lysine–coated slides were quickly thawed and fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 15 minutes. The slides were washed twice in phosphate-buffered solution (PBS) containing 0.1% active diethyl pyrocarbonate for 15 minutes each time, and then in 5× saline sodium citrate (SSC; 1× SSC